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Activation of mTOR controls the loss of TCRζ in lupus T cells through HRES-1/Rab4-regulated lysosomal degradation

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Abstract

Persistent mitochondrial hyperpolarization (MHP) and enhanced calcium fluxing underlie aberrant T-cell activation and death pathway selection in systemic lupus erythematosus. Treatment with rapamycin, which effectively controls disease activity, normalizes CD3/CD28-induced calcium fluxing but fails to influence MHP, suggesting that altered calcium fluxing is downstream or independent of mitochondrial dysfunction. Here, we show that activity of the mammalian target of rapamycin (mTOR), which is a sensor of the mitochondrial transmembrane potential, is increased in lupus T cells. Activation of mTOR causes the over-expression of the Rab5A and HRES-1/Rab4 small GTPases that regulate endocytic recycling of surface receptors. Pull-down studies revealed a direct interaction of HRES-1/Rab4 with the T-cell receptor/CD3ζ chain (TCRζ). Importantly, the deficiency of the TCRζ chain and Lck and compensatory upregulation of the Fcε receptor type I γ chain (FcεRIγ) and Syk, which mediate enhanced calcium fluxing in lupus T cells, was reversed in patients treated with rapamcyin *in vivo*. Knockdown of HRES-1/Rab4 by siRNA and inhibitors of lysosomal function augmented TCRζ protein levels. The results suggest that activation of mTOR causes the loss of TCRζ in lupus T cells through HRES-1/Rab4-dependent lysosomal degradation.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown etiology characterized by T- and B-cell dysfunction and production of anti-nuclear antibodies. Dysregulation of cell death in SLE is thought to play a key role in driving anti-nuclear antibody production, since the source of immunogenic nuclear material is necrotic or apoptotic cells. Nucleosomes are a major immunogen for pathogenic autoantibody-inducing T cells in murine lupus [1]. There is enhanced spontaneous apoptosis of circulating T cells in SLE, which has been linked to chronic lymphopenia [2] and compartmentalized release of autoantigens [3]. Paradoxically, there is decreased activation-induced T cell death in SLE [4–6], which may contribute to persistence of autoreactive cells.

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The mitochondria play crucial roles in activation and death pathway selection in T lymphocytes; however, the mechanistic roles of mitochondria and the underlying metabolic pathways in altered lymphocyte activation and death of SLE patients are incompletely understood [7]. Lupus T cells exhibit mitochondrial dysfunction which is characterized by the elevation of the mitochondrial transmembrane potential ($\Delta\psi_m$) or persistent mitochondrial hyperpolarization (MHP) and consequential ATP depletion, resulting in decrease of activation-induced apoptosis and predisposition of T cells for necrosis [5]. ATP depletion in lupus T cells was recently confirmed by Krishnan et al [8]. We proposed that increased release of necrotic materials from T cells could drive disease pathogenesis by activating macrophages and dendritic cells (DCs) and enhancing their capacity to produce nitric oxide (NO) and interferon α (IFN-α) in SLE [7]. Indeed, DCs exposed to necrotic, but not apoptotic, cells induce lupus like-disease in MRL mice and accelerate the disease of MRL/lpr mice [9].

Enhanced T cell activation-induced Ca^{2+} fluxing has been identified as a central defect in abnormal activation and cytokine production by lupus T cells [10]. Induction of MHP and mitochondrial biogenesis by NO augments cytoplasmic Ca^{2+} levels and regenerates the enhanced rapid Ca^{2+} signaling profile of lupus T cells [11]. Dysregulation of signaling through the T cell receptor has also been shown to be a critical determinant of abnormal calcium fluxing in SLE [12;13]. The T cell receptor/CD3 ζ chain (TCRζ) expression is diminished in SLE T cells and it is functionally replaced by the Fcε receptor type I γ chain (FcεRIγ), a protein normally found in other cell types[14]. T cell receptor signaling with FcεRIγ and its adaptor protein Syk is associated with elevated calcium fluxing, but only in the absence of TCRζ [12]. It has been shown that forced expression of TCRζ is sufficient to reduce calcium influx in SLE T cells to healthy control levels, illustrating the critical role regulation of TCRζ plays in the SLE phenotype [12].

The mammalian target of rapamycin (mTOR), which is a sensor of the $\Delta\psi_m$ in the outer mitochondrial membrane in T cells [15], may serve as a checkpoint between MHP and enhanced Ca^{2+} release [16]. While mTOR is highly conserved and controls protein translation and other metabolic pathways in all mammalian cells [17], it plays a particularly critical role in T cell activation. Inhibition of mTOR by rapamycin has been shown to block T cell function specifically, leading to the therapeutic introduction of rapamycin for preventing organ transplant rejection [18]. Treatment with rapamycin resulted in markedly improved disease activity in SLE patients resistant or intolerant to conventional immunosuppressants [19]. MHP persisted while CD3/CD28-induced calcium fluxing was normalized in T cells of rapamycin-treated patients, suggesting that altered calcium fluxing is downstream or independent of mitochondrial dysfunction [19]. mTOR has recently been localized to endosomes carrying Rab7 [20] that controls the traffic between early and late endocytic organelles [21].

The classical model of signaling involves membrane-spanning receptors that, after binding an extracellular ligand at the cell surface, activate secondary messengers in the cytosol, enabling the spread of the signal into the nucleus. The signaling machinery can achieve a high order of regulation by exploiting the compartmentalization and functional specialization of the endocytic pathway [22;23]. Endosomal transport is tightly regulated by the Ras-like small Rab GTPases [24]. Although the role of receptor recycling and endosomal trafficking in the immune system is largely unknown, it is likely to be significant based on a few established models. A dominant-negative form of Rab4, Rab4N121I, inhibited antigen-presentation by a B-cell line to a T-cell hybridoma [25]. In T cells, CD3-induced Ca^{2+} fluxing and proliferation are enhanced in transgenic mice expressing dominant-negative Rab5A [26]. HRES-1/Rab4 has a dominant influence on expression of CD4, and to a lesser extent on transferrin receptor (TFR), by regulation of their recycling from endosomes in Jurkat and peripheral blood T cells [27]. While the plasma membrane-associated Lck is bound to CD4, an intracellular pool of Lck is associated with TFR-positive recycling endosomes [28;29]. The TCRζ chain is also physically associated

with TFR [30]. mTOR was shown to modulate the recycling of TFR [31] which traffic through HRES-1/Rab4-positive endosomes in Jurkat cells [27]. Therefore, we assessed the role of endosome traffic and Rab proteins in mediating the effect of mTOR on T cell activation.

In the present study, we show that mTOR activity is increased in lupus T cells. Based on whole-transcriptome analysis, we identified a "mitochondrial" gene expression signature in negatively isolated untouched T cells of lupus patients characterized by the over-expression of superoxide dismutase 2 (SOD2), voltage-dependent anion channel 1 (VDAC1), and transaldolase (TAL) and diminished expression of endothelial nitric oxide synthase inhibitory protein (NOSIP), none of which was affected by rapamycin treatment. By contrast, the increased expression of Rab5A and HRES-1/Rab4 small GTPases, that regulate endocytic recycling of receptors, was reversed by rapamycin, suggesting a role for mTOR in eliciting these changes. Along the same line, activation of mTOR was inducible by NO, a key trigger of MHP and mitochondrial biogenesis [32]. In turn, NO-induced stimulation of HRES-1/Rab4 expression was reduced by rapamycin. Thus, NO-dependent MHP lies upstream while enhanced expression of HRES-1/Rab4 lies downstream of mTOR activation in lupus T cells. Further downstream, CD4, Lck, and TCRζ protein levels were depleted while Syk and FcεRIγ levels were augmented in lupus T cells, all of which were reversed in SLE patients treated with rapamycin *in vivo*. Pull-down and confocal microscopy studies revealed a direct interaction of HRES-1/Rab4 with TCRζ. Depletion of TCRζ in lupus T cells was reversed by HRES-1/Rab4 knockdown as well as by inhibition of lysosomal function *in vitro*, indicating that activation of mTOR causes the loss of TCRζ through HRES-1/Rab4-dependent lysosomal degradation.

RESULTS

Detection of the mitochondrial and endocytic recycling gene expression signatures in lupus T cells

The signaling network underlying T cell dysfunction was investigated by a series of microarray analysis of gene expression and parallel flow cytometry of mitochondrial function in 15 female lupus patients relative to 17 female controls. Log2-based normalized expression levels of genes present in one of the two study groups (lupus and control cells) were compared with ANOVA. P values were adjusted for multiple comparisons with the Holm formula using Genespring software. Similar to previous findings, mitochondrial mass, mitochondrial transmembrane potential, cytoplasmic Ca^{2+}, and NO production were enhanced in lupus T cells (Fig. S1). Among 10 of 15 lupus patients, that have not been exposed to prednisone or cytotoxic drugs (Table S1), expression of 117 genes was altered. 82 of these genes had identified functions. Although many genes participate in multiple signaling networks, 22 were involved in metabolic control of $\Delta\psi_m$, 19 in Ca^{2+} signaling, 12 were involved in cytokine/interferon pathway confirming earlier results [33–35], 6 in programmed cell death, 8 were transcription factors, 5 were surface receptors/adaptor proteins, and 9 in small GTPase-mediated intracellular traffic (Table S2). While lupus T cells exhibit persistent MHP, exposure of normal human T cells to CD3/CD28 co-stimulation or NO elicit transient MHP [32]. To distinguish the impact of SLE from that of T-cell activation, the influence of CD3/CD28 co-stimulation and NO on gene expression was also assessed in negatively isolated "untouched" T cells of four healthy female donors. Expression of 329 genes was increased and expression of 173 genes was reduced by CD3/CD28 co-stimulation (Fig. S2). Expression of 50 genes was increased and expression of 204 genes was reduced by exposure to NO donor NOC-18 (Fig. S3). Direction and extent of CD3/CD28 or NO-induced changes in expression of 48 genes correlated with differences observed in patients with SLE. Alternatively, altered expression of 34 genes could not be attributed to CD3/CD28 or NO and thus, they may reflect the disease process in SLE. Expression of 25/34 'lupus-specific' genes correlated with one or more parameter of mitochondrial dysfunction in T cells; while 13/48 genes coordinately regulated by CD3/CD28

stimulation correlated with mitochondrial dysfunction (Table S2; chi-square: 17.27; $p < 0.0001$). This analysis suggested that lupus-specific changes in gene expression, rather than genes coordinately regulated by CD3/CD28, are more closely linked to mitochondrial dysfunction. Among the 34 genes significantly altered in negatively isolated T cells, 1.8-fold or more, VDAC1, SOD2, and Rab5A were one of 35 genes found to be elevated and predictive of SLE in each lupus patient (Fig. S3).

The mitochondrial gene expression signature is characterized by over-expression of SOD2, VDAC1, transaldolase, and FKBP12, diminished expression of endothelial nitric oxide synthase interacting protein (NOSIP) and associated with the activation of the mammalian target of rapamycin (mTOR) in lupus T cells

The functional relevance of changes in gene expression was further investigated on the protein level by western blot analysis. As shown in Fig. 1, expression of mitochondrial proteins superoxide dismutase type 2 (SOD2) and voltage-dependent anion channel 1 (VDAC1) were elevated in negatively isolated lupus T cells. Increased SOD2 and VDAC1 protein levels are consistent with increased mitochondrial mass and oxidative stress in lupus T cells [11]. Moreover, the 12 kD FK506-binding protein (FKBP12) and transaldolase (TAL) protein levels were also elevated in lupus T cells (Fig. 1). FKBP12 is the cellular receptor of rapamycin and their complex inhibits activity of mTOR. The mTOR protein is located in the outer mitochondrial membrane and serves as a sensor of the $\Delta\psi_m$ [15] and regulator of metabolic pathways [36]. In turn, TAL regulates the $\Delta\psi_m$ through controlling NADPH production by the pentose phosphate pathway and thus maintaining GSH in its reduced form [37;38]. Enzymatic activity of TAL was increased in monocyte-depleted PBL (>80% CD3+ T cells) of 32 patients with SLE (20.40 ± 1.41 mU/mg protein) as compared with 12 control donors (14.80 ± 1.17 mU/mg protein; p=0.004). Expression of NOSIP, which inhibits activity of eNOS, was reduced in lupus T cells (Fig. 1). Diminished NOSIP expression was associated with elevated production of NO (Fig. S1), a key factor eliciting mitochondrial dysfunction in lupus T cells [11].

mTOR is associated with the outer mitochondrial membrane and senses mitochondrial dysfunction and changes of the $\Delta\psi_m$ in T cells [15]. With a focus on mitochondrial dysfunction, we began to utilize rapamycin for treatment of SLE patients resistant or intolerant to conventional medications. In patients treated with rapamycin, we observed normalization of CD3/CD28-induced Ca^{2+} fluxing and persistence of MHP [19], indicating that increased Ca^{2+} fluxing is downstream or independent of MHP in the pathogenesis of T-cell dysfunction in SLE. mTOR associates with two protein complexes, TORC1 and TORC2 [17] in which mTOR is bound to the protein partner raptor or rictor, respectively [17]. TORC1 controls cell growth via phosphorylation of two key substrates, S6K1 and 4E-BP1, which is inhibited by rapamycin [17]. Phosphorylation of S6, a 40S ribosomal protein drives protein synthesis and mRNA translation. The immunosuppressive properties of rapamycin have been attributed to the blocking of TORC1 that is required for transducing T cell activation initiated by cytokines [39]. TORC2 is required for organization of the actin cytoskeleton and it is rapamycin-insensitive. Protein levels of mTOR, raptor, rictor (not shown), S6K, and 4E-BP1 were similar in lupus and control T cells (Fig. 2). However, phosphorylation levels of mTOR substrates S6K1 and 4E-BP1 were increased 2.5-fold (p=0.023) and 2.7-fold in lupus T cells (p=0.014), respectively, which were reversed in rapamycin-treated lupus patients (Fig. 2). These findings suggest that mTOR kinase activity is increased in lupus T cells and it is reversed by rapamycin treatment.

In accordance with the persistence of increased mitochondrial mass and MHP [19], elevated expression of SOD2, VDAC1, TAL (not shown), and FKBP12 and diminished expression of NOSIP were unaffected in T-cells of rapamycin-treated patients (Fig. 2).

Activation of endocytic recycling pathway is characterized by over-expression of Rab5A and HRES-1/Rab4

Microarray analysis of gene expression also showed increased Rab5A levels in negatively isolated lupus T cells (Fig. S3. And Table S2). The over-expression of Rab5A was confirmed by western blot studies of lupus T cells (Fig. 3A). Rab5A regulates internalization of surface receptors from the plasma membrane to early endosomes [23]. In contrast, Rab4 is found on endocytic vesicles that recycle cargo from the early endosomes [40]. The predominant isoform of Rab4 in T cells, HRES-1/Rab4 [27], is over-expressed in lupus T cells (Fig. 3A). With respect to β-actin internal control, HRES-1/Rab4 expression was increased 2.14-fold in T cells of 26 lupus patients relative to 23 healthy controls (p= 0.0011) Since genetically enforced over-expression of HRES1/Rab4 down-regulated CD4 in normal T cells [27], we investigated whether CD4 levels were altered in lupus T cells. CD4 protein levels were reduced in negatively isolated untouched lupus T cells by 46% (p = 0.045; not shown). The reduction in CD4 protein levels was not due to diminished numbers of CD4+ T cells since the CD4/CD8 ratio within the CD3 + T-cell compartment was similar between control (3.50; n=10) and lupus donors (3.45; n=21). Moreover, HRES-1/Rab4 protein levels were elevated (3.7-fold, p=0.03; Fig. 3B) while CD4 protein levels were also reduced in negatively isolated CD4+ T cells (by 45%; p=0.003; Fig. 3B) from 26 Caucasian female lupus patients relative to 10 healthy Caucasian female controls. Protein levels of Rab7, which regulates transport between early and late endocytic organelles [21], were similar in T cells of control and SLE donors (not shown).

Enhanced recycling of CD3 and CD4 on lupus T cells

To determine whether increased expression of Rab5A and HRES-1/Rab4 and depletion of CD4 are associated with altered receptor traffic, constitutive recycling of surface receptors was investigated in negatively isolated T cells from 14 SLE patients, 9 healthy and 6 RA controls. The recycling assay was performed after staining the cells with antibodies against CD3ε, CD4, and CD8, then washing the cells and allowing the constitutive internalization and recycling of receptors to take place at 37°C for varying amounts of time. The cells were then restained and the mean fluorescence intensity of the cells, that was assumed to be proportional to the speed at which internal molecules are brought to the cell surface, was determined by flow cytometry. As shown in Fig. 4, recycling of CD3 and CD4 was enhanced on lupus T cells relative to T cells from healthy and RA controls. Surface expression and recycling rates of CD8 were similar in lupus and control T cells (not shown).

Expression of HRES-1/Rab4 is NO-inducible and rapamycin-sensitive

Mitochondrial hyperpolarization and increased mitochondrial biogenesis require exposure of T cells to NO [11]. In accordance with previous findings [41], NOC-18 (300 μM), which is a capable of slowly releasing NO, and H_2O_2 (50 μM) stimulated mTOR activity 5.4-fold (n=7; p=0.005) and 4.5-fold, respectively (n=7; p=0.002; representative western blots are shown in Fig. 5). To determine whether increased expression of HRES-1/Rab4 is induced by NO or the resultant oxidative stress, PBMC were exposed to NOC-18 and H_2O_2. As shown in Fig. 5, expression of HRES-1/Rab4 was induced by 300 μM NOC-18 (n=6; p=0.0003) and 50 μM H_2O_2 (n=7; p=0.019). Inhibition of NO synthase by L-NMMA reduced expression of HRES-1/Rab4 (Fig. 5). In vitro treatment with rapamycin diminished the NO-dependent activation of HRES-1/Rab4 by 55% (n=7, p=0.024; Fig. 5). While exposure of PBMC to IFN-α markedly enhanced the expression of MX1, IFIT1, IFI44, and PRKR, as earlier described [42], IFN-α failed to influence the expression of HRES-1/Rab4 or activity of mTOR, assessed by S6K phosphorylation (data not shown).

Rapamycin treatment in vivo reduces the expression of HRES-1/Rab4 and Rab5A and reverses the loss of CD4, Lck, and TCRζ chain in lupus T cells

mTOR was shown to modulate the recycling of GLUT4 [43;44] and the transferrin receptor (TFR) [31] which traffic through Rab4-positive endosomes in adipocytes [45] and epithelial cells [46], respectively. However, an interaction of mTOR with Rab4 has not been previously demonstrated. Here, we show that over-expression of Rab5A and HRES-1/Rab4 were reduced in T cells of rapamycin-treated lupus patients (Fig. 6). Since HRES-1/Rab4 has a dominant impact on expression of CD4 via controlling its recycling and targets CD4 for lysosomal degradation, we also examined CD4 expression in control donors as well as in SLE patients treated without or with rapamycin. As shown in Fig. 6, CD4 protein levels were reduced in SLE patients and these changes were reversed in T cells from rapamycin-treated patients.

The CD4 co-receptor plays essential roles in formation of the immunological synapse (IS) which is altered in lupus T cells [47;48]. TCR/CD3ζ levels are depleted in lupus T cells which leads to a compensatory enhancement of the Fcε receptor type I γ chain (FcεRIγ) and Syk expression and plays a critical role in enhanced CD3-induced Ca^{2+} fluxing [12]. Lck levels are also reduced in lupus T cells [47;49]. Lck is constitutively associated with the CD4 [28;29;50] and it is brought to the IS via binding to CD4 [51]. As shown previously, HRES-1/Rab4 has a dominant influence on expression of CD4, and to a lesser extent on TFR, by regulation of their recycling from endosomes in Jurkat T cells and primary peripheral blood T cells [27]. Expression of CD4 was reduced in lupus T cells and reversed in rapamycin-treated patients (Fig. 6). Since rapamycin treatment also corrected the enhanced Ca^{2+} fluxing in lupus T cells [19], we investigated whether this was mediated through restoration of Lck and CD3ζ chain expression. In accordance with previous findings [12;14], we detected profoundly diminished Lck and TCRζ levels in lupus T cells (Fig. 7). Depletion of both Lck and TCRζ protein levels was reversed in T cells of rapamycin-treated lupus patients (Fig. 7). While confirming elevated FcεRIγ and Syk protein levels in lupus T cells, we also demonstrated their partial reversal in rapamycin-treated patients (Fig. 7).

HRES-1/Rab4 interacts with the TFR, CD4, CD2AP, and TCRζ

To assess the interaction HRES-1/Rab4 with T-cell surface receptors and adaptor proteins through pull-down studies, we coupled the HRES-1/Rab4-GST fusion protein to GSH-agarose beads [27]. TFR and CD4 were efficiently pulled down by HRES-1/Rab4-GST but not by GST alone in Jurkat cells (Fig. 8A). CD2AP and TCRζ were also pulled down by HRES-1/Rab4-GST but not by GST alone from PBL lysates (Fig. 8B). As controls, CD8 and Rab5A (Fig. 8) as well as transaldolase, mTOR, raptor, rictor, and CD3ε (not shown) did not affinity-purify with HRES-1/Rab4-GST beads. As expected, CD4 and TCRζ were depleted in activated T cells (Fig. 8B). The pull-down of TFR, CD4, and TCRζ was enhanced in the presence of non-hydrolyzable GTPγS, suggesting that these receptors have a higher affinity for the membrane-associated GTP-bound form [46;52].

Co-localization of TCRζ with HRES-1/Rab4 and lysosomes in lupus T cells

Confocal microscopy of negatively isolated resting T cells from healthy controls showed that TCRζ and HRES-1/Rab4 were present in the cell membrane or intracellular vesicles but not in acidified lysosomes stained with lysotracker red (LTR) (Fig. 9). HRES-1/Rab4 partially co-localized with lysosomes in normal T cells. By contrast, the depletion of TCRζ in lupus T cells was associated with the re-distribution of the residual of TCRζ into HRES-1/Rab4-positive intracellular compartments that co-localized with lysosomes (Fig. 9).

Depletion of TCRζ in lupus T cells is reversed by HRES-1/Rab4 knockdown and inhibition of lysosomal function

Over-expression of HRES-1/Rab4 causes lysosomal degradation of CD4 [27]. Following stimulation by antigen or CD3, the TCR/CD3ζ chain was also shown to be degraded in the lysosome [53;54]. To investigate whether HRES-1/Rab4 over-expression contributes to depletion of TCRζ in lupus T cells, we reduced HRES-1/Rab4 protein levels by transfection of siRNA. Knockdown of HRES-1/Rab4 augmented TCRζ protein levels in lupus T cells (Fig. 10A). TCRζ levels were significantly increased (1.54 ± 0.17-fold; p=0.013) by achieving • •50% siRNA-mediated knockdown of HRES1/Rab4 36h after transfection in 5/5 SLE patients with baseline HRES1/Rab4 levels • •2.1-fold over 10 healthy controls. Inhibition of lysosomal function with bafilomycin A1 or folimycin also significantly increased TCRζ levels in lupus T-cells (Fig. 10B).

DISCUSSION

Abnormal activation and death of T lymphocytes has been extensively documented in patients with SLE [7;13] and recently associated with two distinct yet connected phenotypes: MHP [5] and enhanced calcium fluxing following TCR stimulation [55]. We have shown that pretreatment of control T cells with NO was adequate to generate both of these conditions, revealing a causal relationship [11]. Rapamycin, that effectively controlled the disease manifestations in patients with SLE, normalized enhanced calcium fluxing, but not MHP. Rapamycin blocks the activity of mTOR, a protein kinase that senses the $\Delta\psi_m$ [15] and regulates protein translation [17] with a particular selectivity for T cells [15], explaining its use as an immunosuppressant that prevents transplant rejection [39]. Therefore, we determined whether dysfunction of lupus T cells was related to 1) an increased activation state of mTOR, 2) investigated the genetic pathways underlying mitochondrial dysfunction, and 3) delineated the mechanism whereby rapamycin treatment led to the normalization of calcium fluxing.

Microarray analysis of gene expression in whole blood or PBMC has been used to identify a variety of genetic signatures in SLE [56]. However, the previous studies had failed to provide subsets of genes linked to mitochondrial dysfunction of T cells. We chose to perform microarrays on negatively isolated “untouched” T cells, providing a distinct data set from those earlier studies, without elimination of previously described signatures. We verified the existence of the well-described interferon signature of SLE in this subset of cells [33;34;57]. Additionally, we identified two novel transcriptional programs termed mitochondrial and endocytic recycling signatures. The functional relevance of these gene expression signatures was validated on the protein level by western blot analysis and functional studies. Increased expression of the mitochondrial proteins VDAC1 and SOD2 is consistent with increased mitochondrial mass and oxidative stress in lupus T cells [11]. Overexpression of TAL enhanced MHP in Jurkat cells [38] and may also act upstream of MHP of lupus T cells. Reduced expression of NOSIP, which inhibits activity of eNOS and may act upstream of elevated NO production in lupus T cells, was not affected by rapamycin treatment. Thus, TAL and NOSIP control metabolic pathways that regulate $\Delta\psi_m$ and may be regarded as markers of the mitochondrial signature in lupus T cells.

Over-expression of Rab5A and Rab4 which control internalization and recycling, respectively, of surface receptors via early endosomes [23;40], were considered markers of an activated recycling gene expression signature. The predominant isoform of Rab4 in T cells, HRES-1/Rab4, is over-expressed in patients with SLE. In accordance with a dominant impact of HRES-1/Rab4 on the endocytic recycling of CD4 [27], there was an inverse correlation between enhanced HRES-1/Rab4 expression and diminished CD4 expression in negatively isolated CD4 T cells (R=0.45, p=0.041). Of note, over-expression of HRES-1/Rab4 was also inversely correlated with TCRζ protein levels (R=0.66, p=0.0009). These changes in gene expression

were associated with enhanced constitutive recycling of CD3ε and CD4 in lupus T cells relative to healthy and RA disease controls.

We identified the activation of mTOR as a critical checkpoint between the enhanced mitochondrial and receptor recycling gene expression signatures. Such gatekeeper function of mTOR is consistent with its role in 1) sensing mitochondrial dysfunction and changes of the $\Delta\psi_m$ in T cells [15] and 2) modulating the traffic of GLUT4 [43;44] and TFR [31] which are associated with Rab4-positive endosomes in adipocytes [45] and epithelial cells [46], respectively. Activation of mTOR in lupus T cells was inducible by NO, a key trigger of MHP and mitochondrial biogenesis [32]. In turn, NO-induced stimulation of HRES-1/Rab4 expression was reduced by rapamycin. Thus, NO-dependent MHP lies upstream while enhanced expression of HRES-1/Rab4 lies downstream of mTOR activation in lupus T cells.

Rapamycin treatment in vivo reduced the expression of HRES-1/Rab4 and Rab5A and reversed the loss of CD4, Lck, and TCRζ chain and the over-expression of FcεRIγ and Syk in lupus T cells. GST pull-down studies revealed a direct interaction of HRES-1/Rab4 with TFR, CD4, CD2AP, and TCRζ. As controls, CD8, Rab5A, transaldolase, mTOR, raptor, rictor, and CD3ε did not affinity-purify with HRES-1/Rab4-GST beads, suggesting that this small GTPase is specifically associated with recycling endosomes carrying a limited subset of TCR-associated surface proteins, such as CD4 and TFR, the intracellular adaptor CD2AP and the critical signal transducer TCRζ. Previous confocal microscopy studies revealed the co-localization of HRES-1/Rab4 with the TFR and CD4 [27]. Here, we demonstrated that TCRζ and HRES-1/Rab4 were present in the cell membrane or intracellular vesicles but not in lysosomes of negatively isolated resting T cells from healthy controls. HRES-1/Rab4 partially co-localized with lysosomes in normal T cells. By contrast, the depleted TCRζ in lupus T cells was re-distributed into HRES-1/Rab4-positive intracellular compartments that co-localized with lysosomes. The knockdown of HRES-1/Rab4 and inhibition of lysosomal function increased TCRζ levels in lupus T-cells. These observations identified HRES-1/Rab4-dependent lysosomal degradation as a novel mechanism contributing to the critical loss of TCRζ in lupus T cells [10].

Although the lysosomal degradation of the TCRζ chain following stimulation by antigen or CD3 has been proposed by pharmacological inhibitor studies [53;54], the mechanism leading to such loss of TCRζ has not been previously determined. The present data suggest that NO, which is produced during T-cell activation and mediates MHP [32], also activates mTOR, enhances HRES-1/Rab4 protein levels, and promotes the the lysosomal degradation of TCRζ. This study reveals that lysosomal degradation of TCRζ also occurs in untouched lupus T cells due to increased production of NO, activation of mTOR, and augmented expression of HRES-1/Rab4.

Development of lupus is determined by an interplay of large number of genetic factors [58]. Of note, both the eNOS-inhibitor NOSIP and HRES-1/Rab4 are located in lupus susceptibility loci mapped to 19q13.33 [59–61] and 1q42[59;62–65], respectively. We earlier mapped HRES-1 to 1q42 [66] and associated polymorphisms of the HRES-1 long terminal repeat (LTR) region with the development of SLE [67;68]. During this study, reproducible individual variations were observed in the extent of HRES-1/Rab4 overexpression among the 26 patients with SLE (mean 2.14-fold ranging from 0.9 to 6.2-fold) relative to 23 healthy subjects (mean set at 1.0 ranging from 0.45 to 2.16). Variations in gene expression are likely to be influenced by polymorphic haplotypes of the LTR [69] which harbors an enhancer of HRES-1/Rab4 gene transcription [27].

The mechanism by which rapamycin influences HRES-1/Rab4 expression is not clear at present. HRES-1/Rab4 mRNA levels were similar between patients treated with or without rapamycin (data not shown), arguing against an influence on gene transcription. mTOR works to enhance the translation of proteins generally, but it has been well described that some

proteins are up-regulated more than others [70] and HRES-1/Rab4 may be one that is affected specifically. NO induced both mTOR activity and HRES-1/Rab4 expression, while rapamycin inhibited the NO-induced expression of HRES-1/Rab4. This suggested that mTOR activity is necessary for stimulating the expression of HRES-1/Rab4. Our GST pull-down results excluded a direct interaction between mTOR, raptor or rictor and HRES-1/Rab4. Colocalization of Rab7 and mTOR has recently been described in HEK293 and HeLa cells [20]. While Rab4 regulates the recycling of early endosomes, Rab7 controls the traffic between early and late endocytic organelles [21]. Although protein levels of Rab7 were similar in T cells of control and SLE donors (not shown), we observed a partial co-localization of mTOR with Rab7 and Rab7-HRES-1/Rab4 double-positive endosomes both in control and lupus T cells (Fig. S4). Thus, mTOR may co-localize and indirectly interact with HRES-1/Rab4 through intermediary molecules other than raptor or rictor. In summary, the results suggest that 1) activation of mTOR causes the loss of TCRζ through HRES-1/Rab4-dependent lysosomal degradation and 2) blocking of TCRζ degradation may account for the therapeutic efficacy of rapamycin in SLE.

MATERIALS AND METHODS

Human subjects

A total of 44 Caucasian female patients with systemic lupus erythematosus (SLE) were investigated. All patients satisfied the criteria for a definitive diagnosis [71]. Disease activity was assessed by the SLEDAI score[72]. Six patients were treated with rapamycin 2 mg/day (age: 40 ± 8.3 years; SLEDAI: 0.8). Among the 38 remaining SLE patients treated without rapamycin, 28 were receiving prednisone (5–50 mg/day) and immunosupppresive drugs including hydroxychloroquine (400 mg/day), mycophenolate mofetil (3 g/day), cyclosporin A (50–100 mg/day). Their mean age was 36.3 ± 4.3 years, ranging between 18–60; SLEDAI: 1.3 ± 0.9. Ten patients (age: 38.5 ± 6.4) SLEDAI: 4.8 ± 3.8) have been freshly diagnosed and have not been treated wifth prednisone or cytotoxic drugs. These patients and five additional patients that have received prednisone or cytotoxic drugs provided cells for microarray analysis (Table S1). As controls, 23 age-matched healthy female subjects and 8 female patients with rheumatoid arthritis (RA; age: 51.3 ± 6.7 years) [73] were studied. RA patients were treated with methotrexate, cyclosporin A, leflunomide, etanercept, or adalimumab. The study has been approved by the Institutional Review Board for the Protection of Human Subjects.

Separation and culture of peripheral blood lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood on Ficoll-Hypaque gradient. Peripheral blood lymphocytes (PBL) were separated from monocytes by adherence to autologous serum-coated Petri dishes [74]. T cells (>95% CD3+) were negatively isolated from PBMC with Dynal magnetic beads conjugated to IgG antibodies for CD14, CD16 HLA class II DR/DP, CD56 and CD235a; Invitrogen Cat No.113-11D). CD4+ T cells (>98% CD4+) were negatively isolated with magnetic beads conjugated to IgG antibodies for CD8, CD14, CD16, HLA class II DR/DP, CD56, CDw123, and CD235a (Invitrogen Cat No.113-39D). The resultant cell population was resuspended at 10^6 cells/ml in RPMI 1640 medium, supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 • •g/ml gentamicin in 12-well plates at 37°C in a humidified atmosphere with 5% CO_2. In vitro treatments of PBL or negatively isolated T cells was performed H_2O_2 (25 and 50 μM; Sigma, St Louis, MO), (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1,2-diolate diethylenetriamine (NOC-18,150 and 300 μM;Calbiochem, San Diego, CA), rapamycin (50 nM dissolved in DMSO, Cell Signaling Cat# 9904), glutathione (GSH, 10 mM; Sigma), L-N^G-monomethyl arginine citrate (L-NMMA,100 μM; Calbiochem), bafilomycin A1 and folimycin (both from Calbiochem).

Flow cytometric analysis of cell viability, mitochondrial transmembrane potential ($\Delta\psi_m$), mitochondrial mass, cytoplasmic and mitochondrial Ca^{2+} levels, and NO production

Apoptosis was monitored by staining with annexin V (annexin V-Alexa-647, Invitrogen) in parallel with monitoring $\Delta\psi_m$, mitochondrial mass, ROI, NO, and Ca^{2+} levels as well as expression of surface antigens, as described in the Supplementary Methods Section.

Microarray analysis of gene expression

RNA was extracted from monocyte-depleted PBL (>80% CD3+ T cells) or untouched T cells (>95% CD3+ T cells negatively isolated with Dynal kit). Biotinylated cRNA was produced by in vitro transcription and hybdridized to Affymetrix HG-U133_Plus_2 chips with 54,675 probe sets, as earlier described [75;76]. Log2-based normalized data on genes present in one of the two study groups (lupus and control PBL or lupus and control T cells) were compared with twoway ANOVA. P values were adjusted for multiple comparisons with the Holm formula. Lupus-predictor genes were identified with the k-nearest-neighbor method (Genespring software, version 7.3, Agilent Inc., Santa Clara, CA), as described in the Supplementary Methods section.

Western blot analysis

Whole cell protein lysates were resuspended in SDS-PAGE sample buffer (2×10^5 cells per 10 μl), separated on a 12% SDS-polyacrylamide gel, blotted to nitrocellulose, and probed with primary rabbit antibodies directed to HRES-1/Rab4 or β-actin, as previously described [27]. Primary antibodies to SOD2, VDAC1, FKBP12, transaldolase, NOSIP, p70 S6 kinase (S6K), phospho-S6K (pS6K), 4E-BP1, phospho-4E-BP1, raptor, rictor, mTOR, CD2AP, TCRζ, CD4, CD8, FcεRIγ, p56 Lck, Syk, Rab5A, and Rab7 as well as peroxidase-conjugated secondary antibodies and detection by chemiluminescence using a Kodak Image Station 440CF are described in the Supplementary Methods section.

Detection of the interferon signature by real-time quantitative PCR

RNA was reverse-transcribed to cDNA and amplified with primers specific for the interferon-inducible genes MX1, IFIT1, IFI44, PRKR, and housekeeping gene control hypoxanthine guanine phosphoribosyltransferase 1, as described earlier [42].

Confocal microscopy

To label acidic lysosomal compartments, negatively isolated T cells were incubated at 37°C with 5uM LysoTracker Red (Invitrogen cat #: L-7528) for 30 minutes in complete RPMI. Cells were washed twice and resuspended in RPMI and allowed to adhere to poly-L-lysine-coated coverslips for 10 minutes at room temperature, followed by fixation in a 4% paraformaldehyde-phosphate buffered saline solution. The cells were permeabilized with a 0.1% saponin/1% fetal bovine serum/Hank's balanced salt solution mixture for 30 minutes. Primary antibodies were directly conjugated with fluorochromes Alexa-488, Alexa-555, or Alexa-647 and applied to permeabilized cells for 45 minutes, washed twice with permeabilization buffer and twice with Hank's balanced salt solution, mounted on slides, and visualized under a Zeiss 510 LSM Meta confocal microscope, as described in the Supplementary Methods section.

Prokaryotic expression of recombinant HRES-1/Rab4 protein and pull-down assays

Full-length HRES-1/Rab4 protein was expressed as a fusion protein with glutathione S-transferase (GST), as earlier described [27]. Cell lysates were prepared from Jurkat cells and peripheral blood lymphocytes (PBL). Supernatants were incubated with 5 μg of HRES-1/Rab4-GST-bound agarose beads or 2.6 μg of GST-bound control beads (~100 pmol of each fusion protein) for 2 h at 4°C with and without 1 mM GTPγS (Sigma). The beads were pelleted,

washed in lysis buffer, and analyzed by SDS-PAGE and Western blot, as described in the Supplementary Methods section.

Transfection of siRNA

Up to 10^7 freshly isolated lymphocytes were electroporated with siRNA specifc for HRES1/Rab4 nucleotides 377–399 or scrambled siRNA, using the Nucleofector protocol for primary human T cells (Amaxa, Gaithersburg, MD). Expression of HRES-1/Rab4 and TCRζ relative to actin was assayed by western blot 6h, 12h, 24h, 36 h, 48h, and 60h after transfection, as described in the Supplementary Methods section.

Recycling assay

Negatively isolated T cells were stained on ice with APC-Cy7-conjugated antibody CD3 (Becton Dickinson/BD Cat# 557832), Alexa 647-conjugated antibody to CD4 (BD Cat# 557707), and PE-conjugated antibody to CD8 (BD Cat# 555367) for 30 minutes, then washed 3 times in ice cold RPMI 1640 medium. The cells were resuspended in warm medium and incubated at 37°C for 3 hours, removing samples at 30 minute intervals and placing them on ice. At the conclusion, the cells were restained with the original antibodies on ice for 30 minutes, then washed 3 times with ice-cold RPMI and analyzed by flow cytometry. Recycling is calculated by the increase in mean fluorescence intensity at given time points over the baseline (time 0 sample) kept on ice after initial staining throughout the assay. The initial antibody incubation step was done with or without 50ug/mL cycloheximide without affecting the results obtained.

Statistical Analysis

Statistical analyses were performed using Prism Version 3.0 for Windows (GraphPad Software, San Diego, CA). Data were expressed as the mean ± standard error of the mean (SE) of individual experiments. Changes were considered significant at p value < 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

FcεRIγ, Fcε receptor type I γ chain
FKBP12, 12 kD FK506-binding protein
GST, glutathione-S-transferase
IFN-α, interferon α
L-NMMA, L-N^G-monomethyl arginine citrate
LTR, Lysotracker Red
MHP, mitochondrial hyperpolarization
mTOR, mammalian target of rapamycin
NO, nitric oxide
NOC-18, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate diethylenetriamine
NOSIP, endothelial nitric oxide synthase inhibitory protein
SLE, systemic lupus erythematosus

SOD2, superoxide dismutase 2
TAL, transaldolase
TCRζ, TCR/CD3ζ chain
TFR, transferrin receptor
VDAC1, voltage-dependent anion channel 1
$\Delta\psi_m$, mitochondrial transmembrane potential

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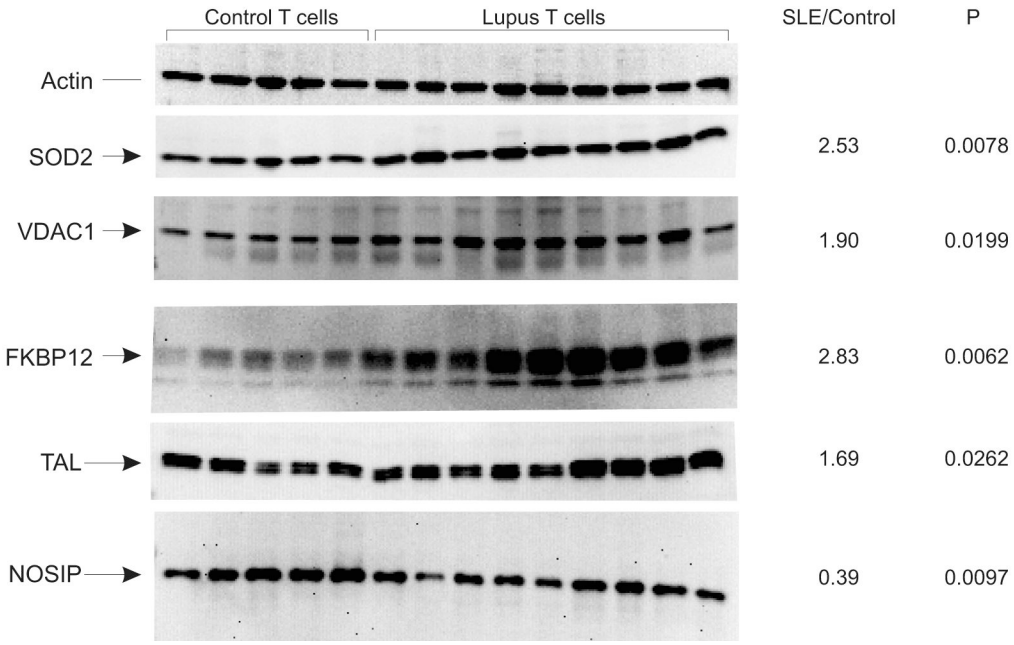

Fig. 1.

Western blot detection of SOD2, VDAC1, FKBP12, TAL, and NOSIP in "untouched" T cells of female patients with SLE and age-matched healthy female controls isolated with the negative T-cell isolation kit (Dynal, Lake Success, NY). SOD2 was detected with rabbit antibody from Santa Cruz (Cat No SC-30080). VDAC1 was detected with goat antibody from Santa Cruz (Cat No SC-8828. FKBP12 was detected with a monoclonal antibody from BD Biosciences (Cat No 554091). Transaldolase was detected with antibody 170 [77]. NOSIP was detected with rabbit antibody [78]. Actin was detected with a monoclonal antibody from Chemicon (Cat No 1501R). The expression of SOD2, VDAC1, FKBP12, TAL, and NOSIP relative to actin was determined in each donor by automated densitometry with Kodak Image Station 440CF. Representative blots are shown. The p values reflect fold changes in protein levels between 14 SLE patients and 10 controls compared with two-tailed t-test.

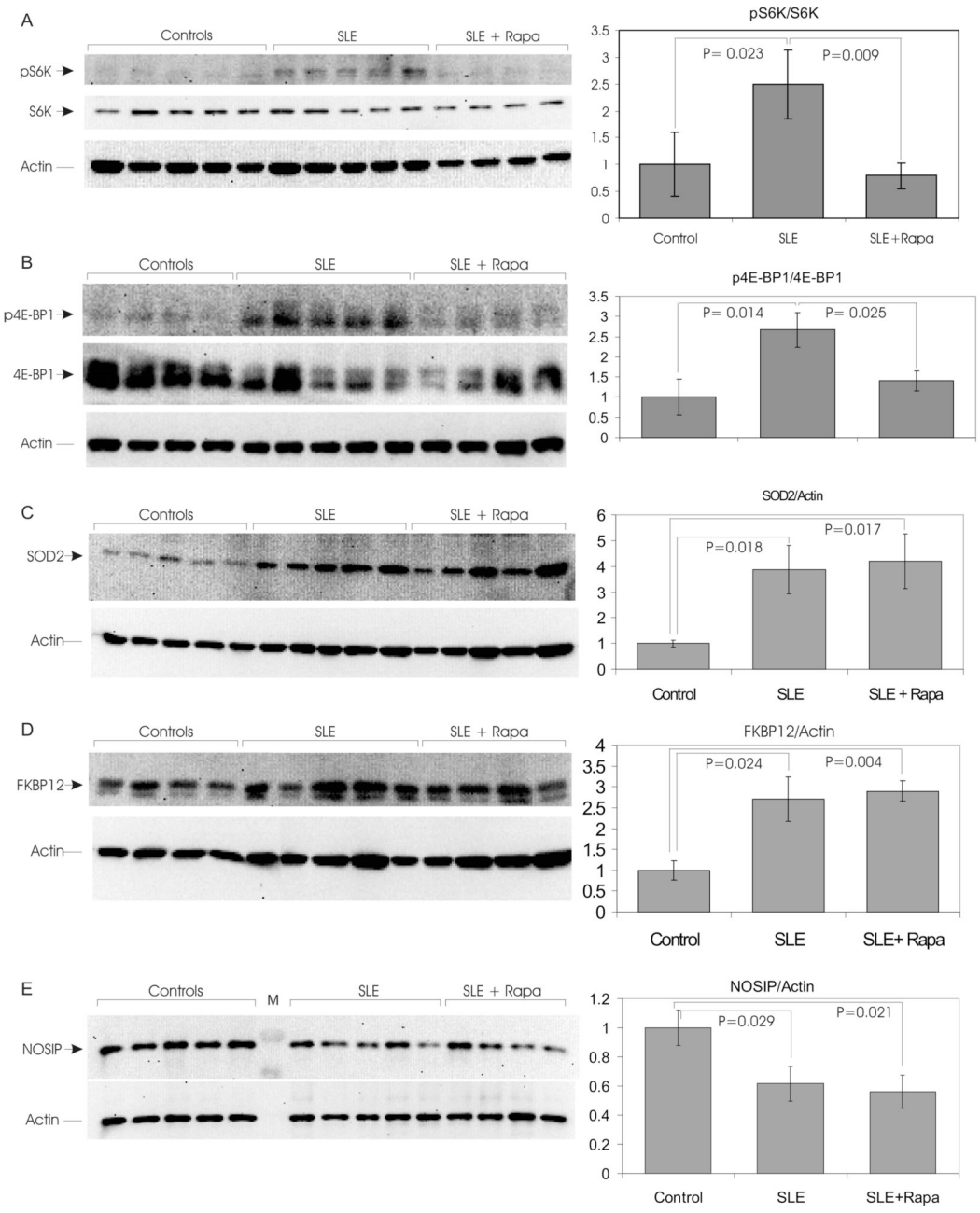

Fig. 2.

Activation of the mammalian target of rapamycin (mTOR) in lupus T cells. A, Western blot analysis of mTOR substrate p70 S6 kinase phosphorylation. Protein lysates of negatively isolated naïve T cells from healthy controls (Controls) and SLE patients treated without (SLE) or with 2mg/day rapamycin (SLE + Rapa) were analyzed by Western blot using antibodies against p70 S6 kinase (S6K) (Santa Cruz, Cat No sc-8418), phospho-S6K (pS6K) (Cell Signaling, Cat#9206) and actin (Chemicon, Cat# MAB1501). Diagram shows fold changes of the phosphorylated S6 kinase signal relative to total S6K. Data represent mean ± SE. B, Western blot analysis of mTOR substrate 4E-BP1 phosphorylation. Phospho-4E-BP1 (Cell Signaling, Cat#2855) and 4E-BP1 were detected rabbit antibodies (Cell Signaling, Cat#9644). C, Effect

of rapamycin on SOD2 expression, detected with rabbit antibody from Santa Cruz (Cat No SC-30080), using the samples analyzed in panel A. D, Effect of rapamycin on FKBP12 expression, detected with a monoclonal antibody from BD Biosciences (Cat No 554091), using the samples analyzed in panel A. E, Effect of rapamycin on NOSIP expression, detected with a rabbit antibody [78], using the samples analyzed in panel A.

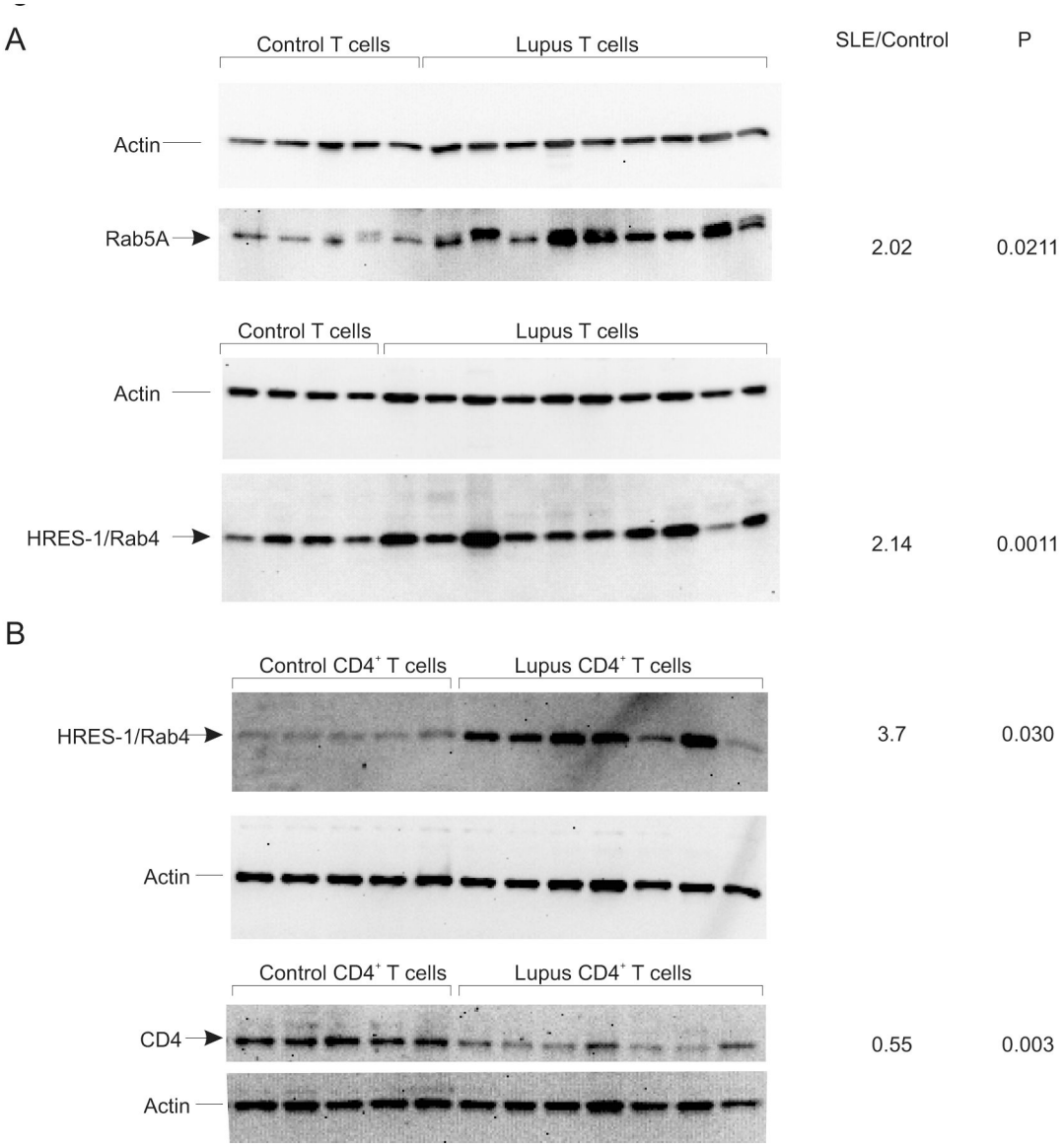

Fig. 3.

A) Western blot detection of Rab GTPases Rab5A and HRES-1/Rab4 in negatively isolated T cells of female SLE and control subjects. Rab5A was detected with rabbit antibody SC 309 (Santa Cruz). HRES-1/Rab4 was detected as earlier [27]. The expression of HRES-1/Rab4 or CD4 relative to actin was determined in each donor by automated densitometry with Kodak Image Station 440CF. The p values reflect fold changes in protein levels between 14 SLE patients and 10 controls compared with two-tailed t-test. B) Western blot detection of HRES-1/Rab4 and CD4 in negatively isolated CD4+T cells of female Caucasian SLE patients and age-matched female Caucasian controls.

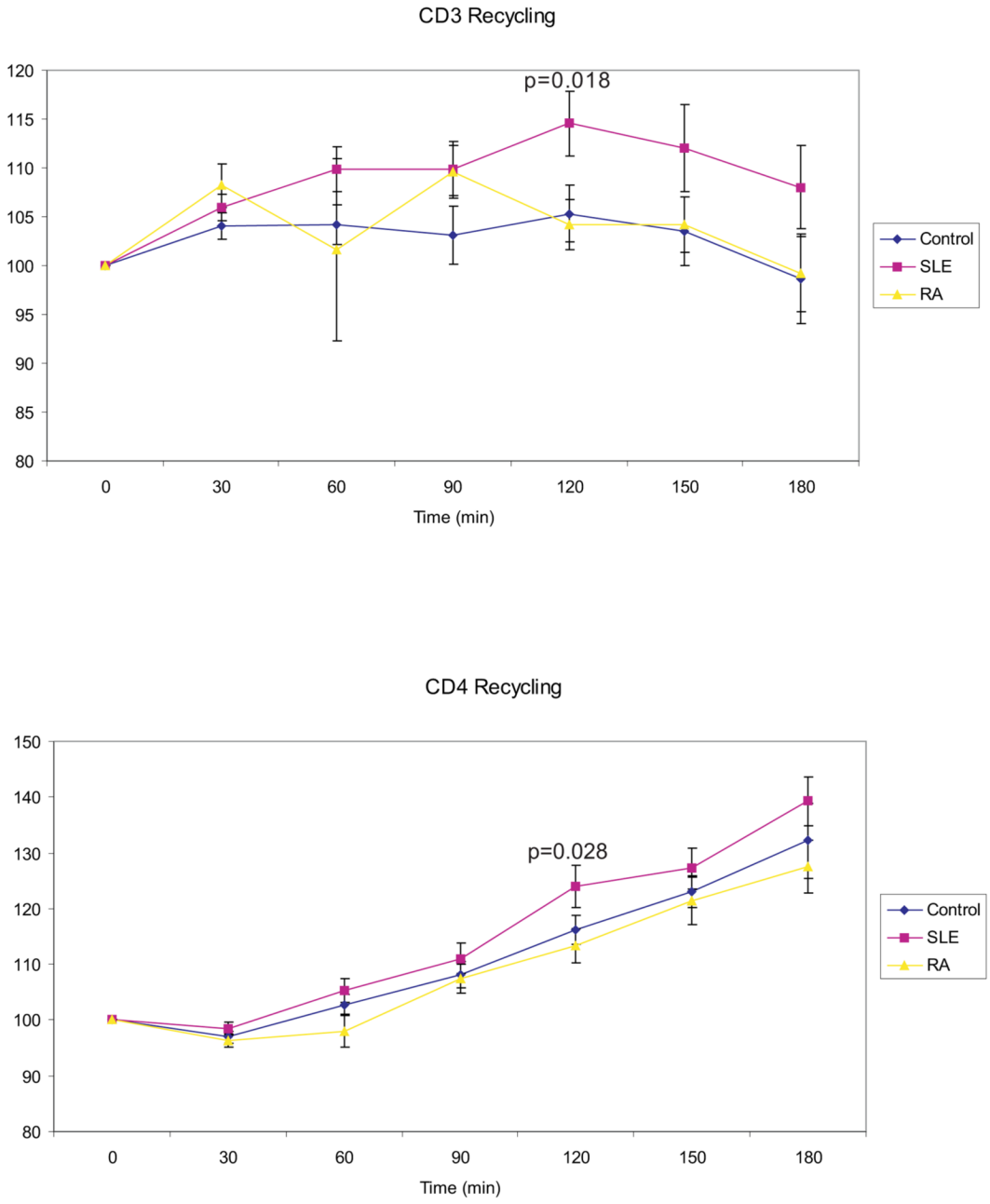

Fig. 4.

Enhanced recycling of CD3 and CD4 of lupus T cells. Negatively isolated T cells from of 14 SLE patients, 9 healthy and 6 RA controls were stained on ice with APC-Cy7-conjugated antibody CD3 (BD Cat# 557832), Alexa 647-conjugated antibody to CD4 (BD Cat# 557707), and PE-conjugated antibody to CD8 (BD Cat# 555367) for 30 minutes (not shown), then washed 3 times in ice cold RPMI 1640 medium. The cells were resuspended in warm medium and incubated at 37°C for 3 hours, removing samples at 30 minute intervals and placing them on ice. At the conclusion, the cells were restained with the original antibodies on ice for 30 minutes, then washed 3 times with ice cold RPMI and analyzed by flow cytometry. The degree

of recycling is given by the increase in mean fluorescence intensity in later time points over the baseline (time 0 sample, kept on ice after initial staining throughout the recycling assay).

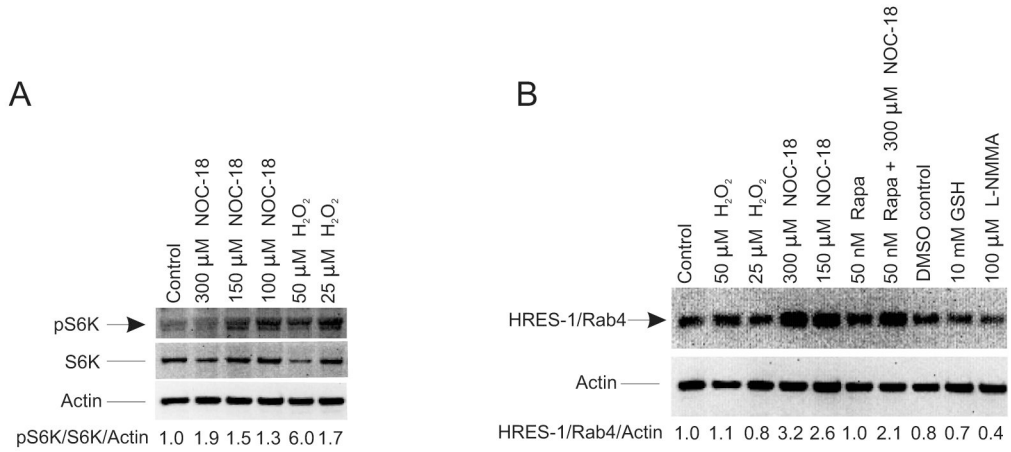

Fig. 5.

Effect of NO and H_2O_2 on mTOR activity and expression of HRES-1/Rab4 in PBMC. A, Effect of 24 h treatment with NO, released by NOC-18 (100, 150 and 300 μM), and H_2O_2 (25 and 50 μM) on mTOR activity assessed by pS6K/S6K/actin ratio. B, Effect of 24 h treatment with NO, released by NOC-18 (150 and 300 μM), and H_2O_2 (25 and 50 μM), rapamycin (50 nM, dissolved in 0.1% DMSO), DMSO alone (0.1%), GSH (10 mM) and L-NMMA (100 μM) on expression of HRES-1/Rab4 relative to actin. Data represent six independent experiments.

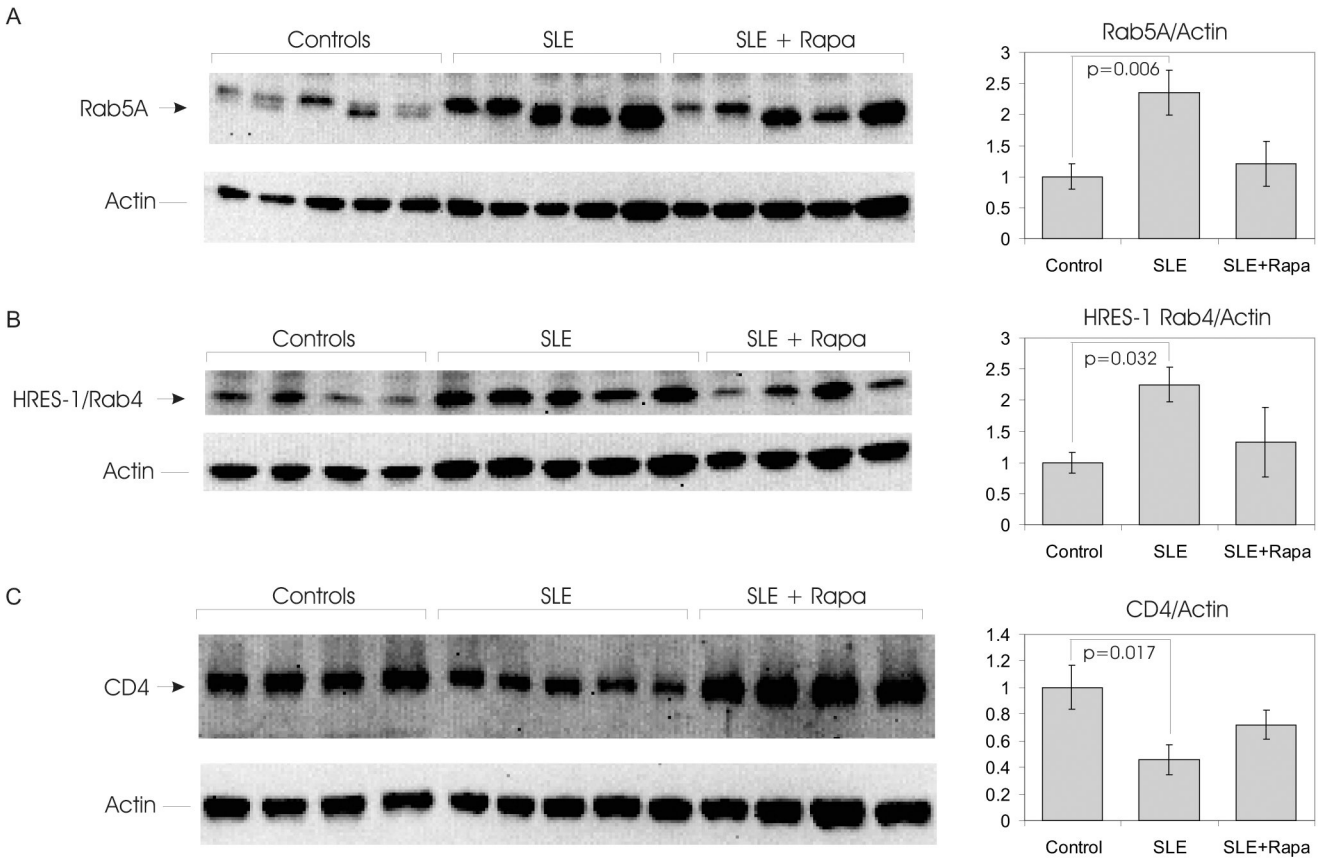

Fig. 6.

Expression of Rab5A, HRES-1/Rab4, and CD4 relative to actin in healthy controls (Controls) and SLE patients treated without (SLE) or with 2mg/day rapamycin (SLE + Rapa). Protein lysates of negatively isolated naïve T cells were analyzed by Western blot. P values < 0.05 reflecting fold changes in protein levels are shown.

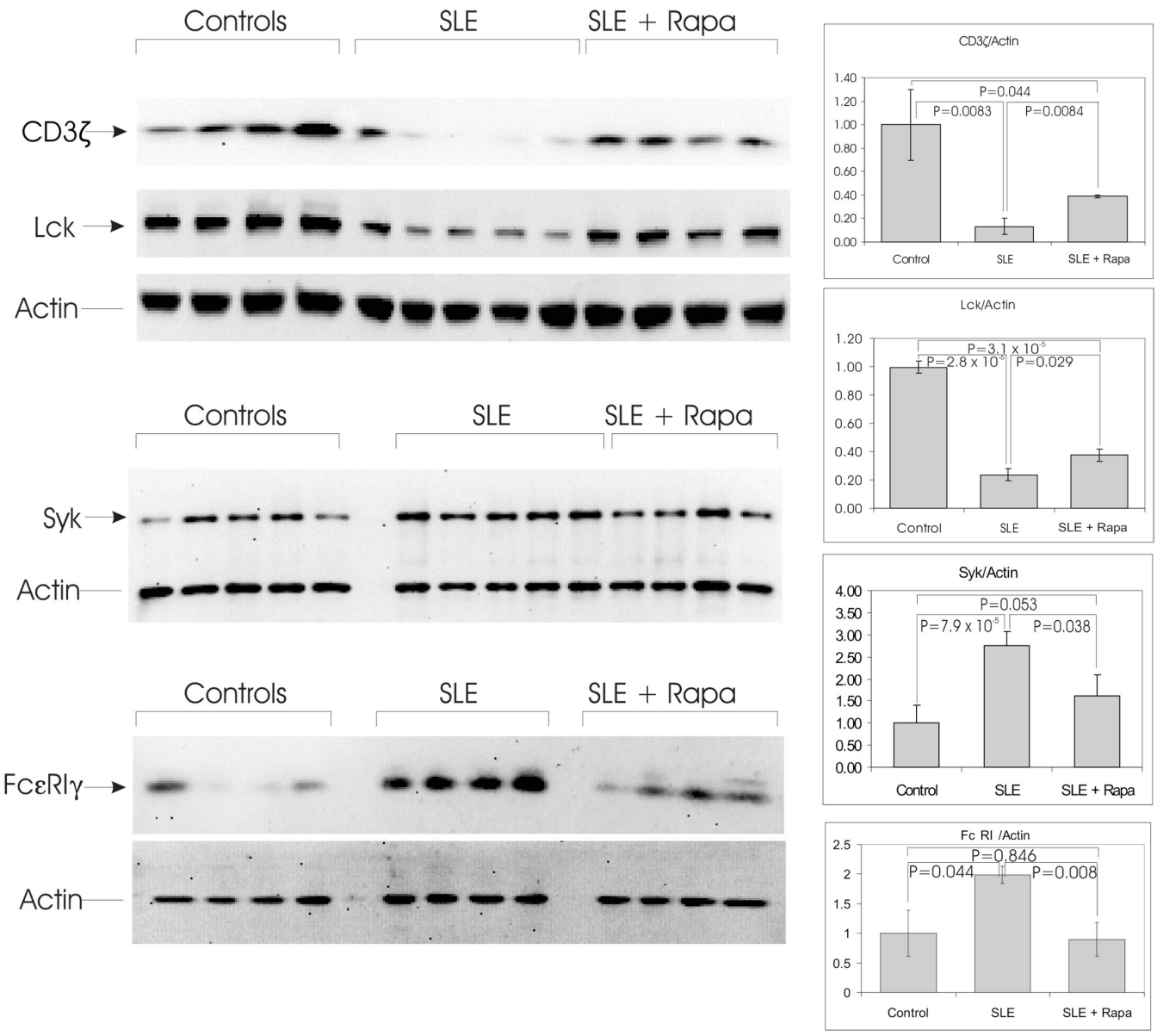

Fig. 7.

Expression of TCR/CD3ζ, Lck, FcεRIγ, and Syk in negatively isolated naïve T cells from healthy controls (Controls) and SLE patients treated without (SLE) or with 2mg/day rapamycin (SLE + Rapa). Protein lysates were analyzed by Western blot. P values < 0.05 reflecting fold changes in protein levels are shown.

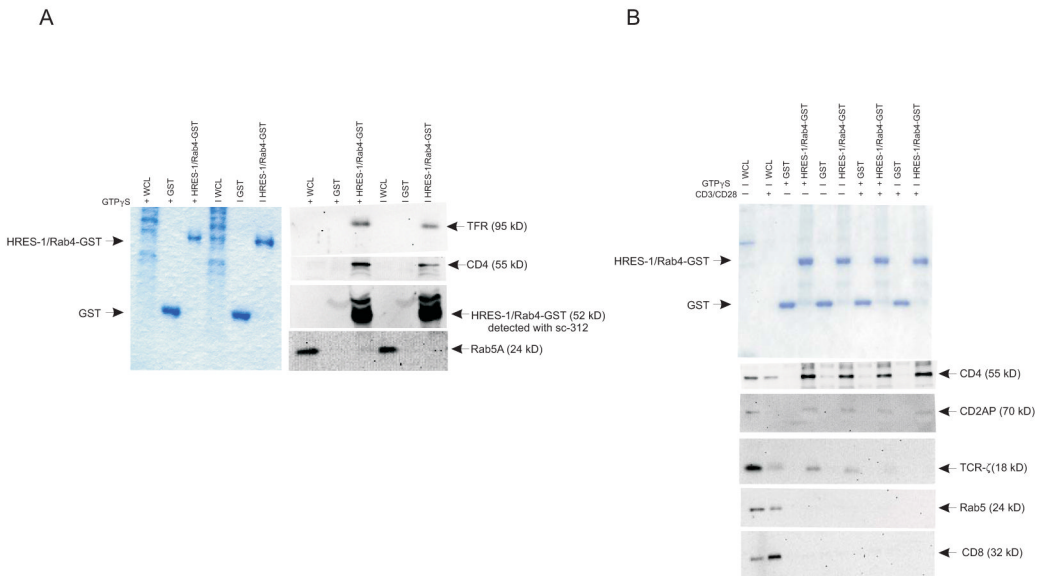

Fig. 8.

Interaction of HRES-1/Rab4 with T-cell surface and adaptor proteins. A, Direct interaction of the TFR and CD4 with HRES-1/Rab4 in Jurkat cells. 10^7 Jurkat cells were lysed in 1% NP-40, 10% glycerol, 200 mM NaCl, 5 mM $MgCl_2$, 50 mM Tris pH 8.0, 1 mM PMSF, 1 mM sodium orthovanadate, 20 mM NaF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin for 30 min at 4°C and the supernatant was obtained by centrifugation at 14,000 × g for 20 min at 4°C. 3 ml of the supernatant was pre-cleared with 1 ml of swollen GSH-agarose beads. 500 μl of pre-cleared supernatant was incubated with 5 μg of HRES-1/Rab4-GST-bound agarose beads or 2.6 μg of GST-bound control beads (~100 pmol of each fusion protein) for 2 h at 4°C with and without 1 mM GTPγS. The beads were pelleted at 500 × g for 5 min at 4°C, washed twice in lysis buffer, resuspended in Laemmli buffer, and analyzed by SDS-PAGE stained with Coomassie brilliant blue (left panel) and by Western blot with the indicated antibodies (right panel). B, Pull-down of CD4, CD2AP, and TCRζ, but not CD8 or Rab5A, from PBL lysates prepared as described for Jurkat cells using agarose beads coupled to HRES-1/Rab4-GST. Antibodies to CD2AP (sc-25272), TCR/CD3ζ (sc-1239), CD8 (sc-53212) and Rab5A (sc-28570) were obtained from Santa Cruz. The results represent four independent experiments.

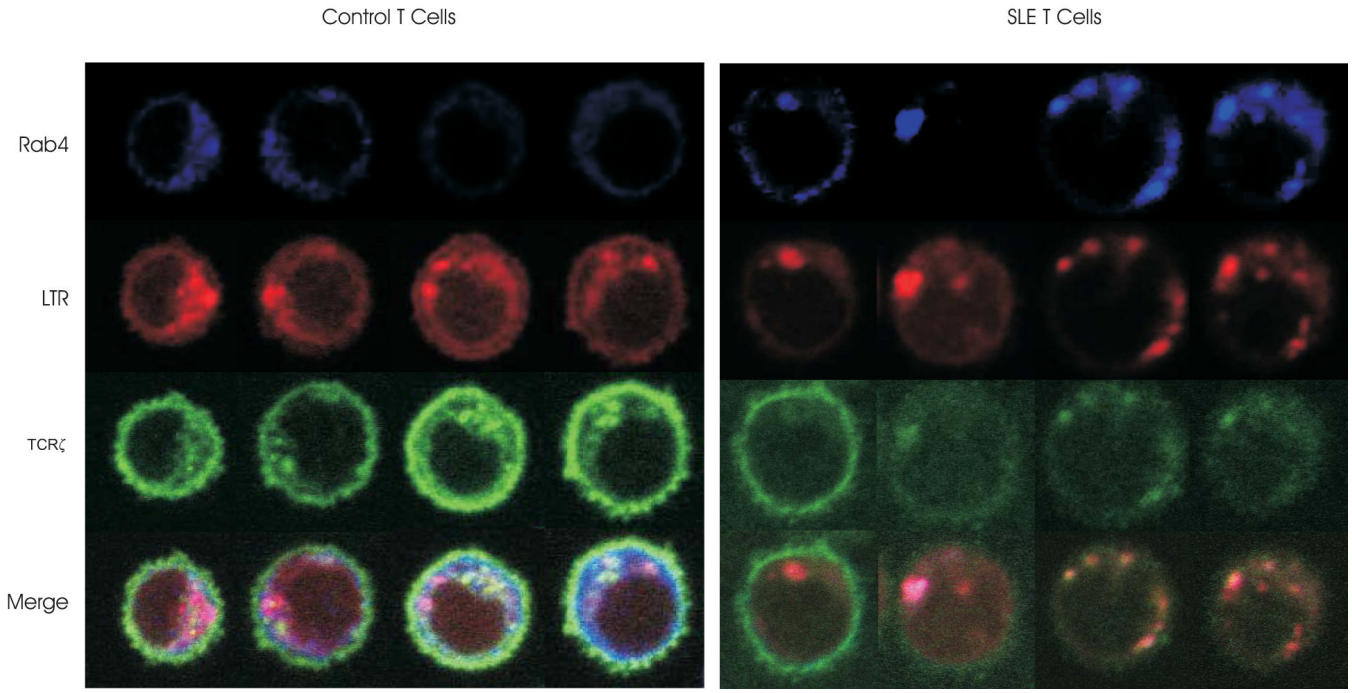

Fig. 9.

Localization of TCRζ, HRES-1/Rab4, and lysosomes in negatively isolated resting T cells from healthy controls and lupus patients using confocal microscopy. TCRζ was visualized with antibody sc-1239 directly conjugated to Alexa-488 (green). HRES-1/Rab4 was detected with antibody sc-312 directly conjugated with Alexa-647 (blue). Lysosomes were detected with Lysotracker Red (LTR). In control T cells, TCRζ and HRES-1/Rab4 were present in the cell membrane or intracellular vesicles but not in lysosomes stained with LTR. In lupus T cells, TCRζ was found predominantly in HRES-1/Rab4-positive intracellular membrane clusters that co-localized with lysosomes. Green color intensities (lower right 8 panels) were enhanced to visualize the localization and compensate for lower expression of TCRζ in lupus T cells.

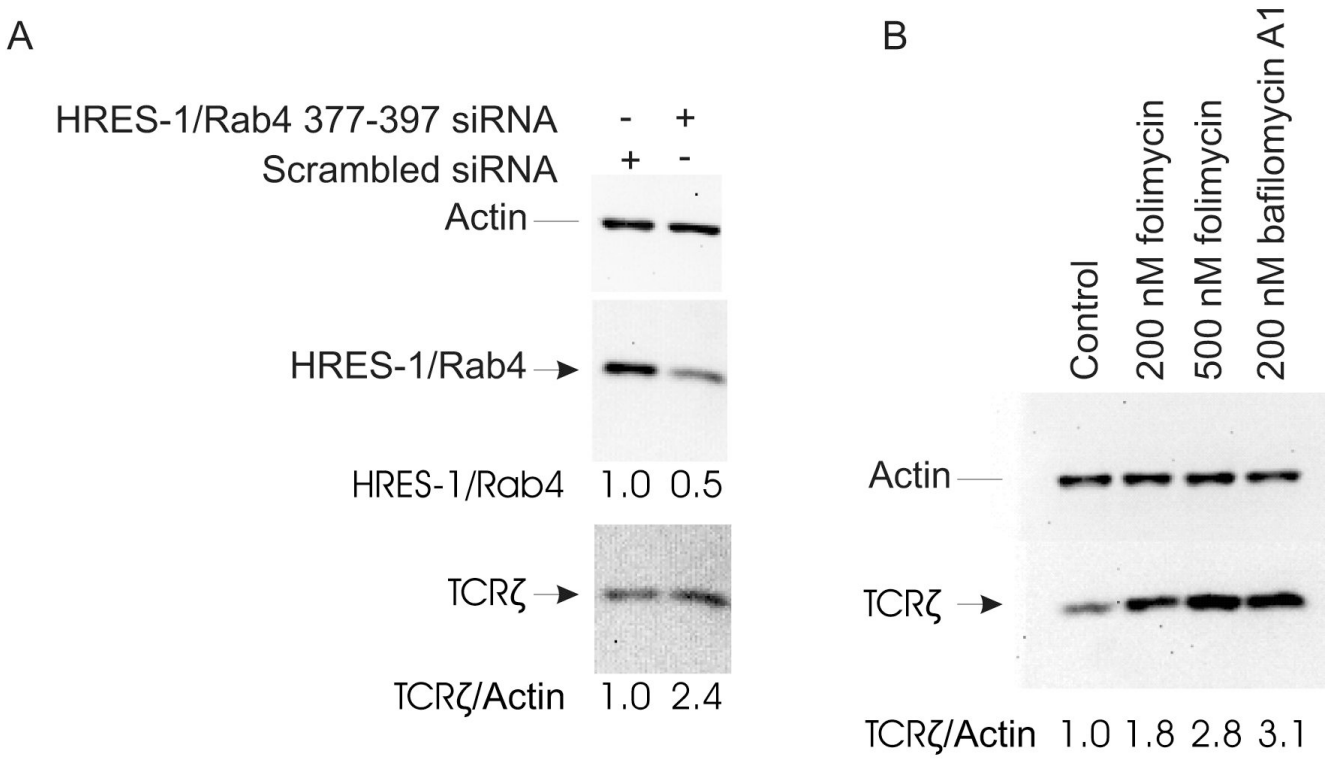

Fig. 10.

Reversal of TCR/CD3ζ depletion in lupus T cells by down-regulation of HRES-1/Rab4 expression and inhibitors of lysosomal function. A, Effect of siRNA-mediated knock-down of HRES-1/Rab4 on TCRζ protein levels in lupus T cells. 10^7 cells were electroporated with siRNA specifc for HRES1/Rab4 nucleotides 377–399 using the Nucleofector protocol for primary human T cells (Amaxa, Gaithersburg, MD) and assayed 36 h later by western blot. Rab4 and TCRζ were detected with antibodies sc-312 and sc-1239. B, Effect of lysosomal inhibitors bafilomycin A1 and folimycin on TCRζ levels of lupus T-cells. The results represent studies of 5 lupus patients.